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Batch and Continuous Culture Kinetics for Production of Carotenoids by β-Ionone-Resistant Mutant of *Xanthophyllomyces dendrorhous*

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Abstract A β-ionone-resistant mutant strain isolated from the red yeast *Xanthophyllomyces dendrorhous* KCTC 7704 was used for batch and continuous fermentation kinetic studies with glucose media in a 2.5-l jar fermentor at 22°C and pH 4.5. The kinetic pattern of growth and carotenoid concentration in the batch fermentations exhibited a so-called mixed-growth-associated product formation, possibly due to the fact that the content of intracellular carotenoids depends on the degree of physical maturation toward adulthood. To determine the maximum specific growth rate constant (μ_m) and Monod constant (k_s) for the mutant, glucose-limited continuous culture studies were performed at different dilution rates within a range of 0.02–0.10 h^{-1}. A reciprocal plot of the steady-state data (*viz.*, reciprocal of glucose concentration versus residence time) obtained from continuous culture experiments was used to estimate a μ_m of 0.15 h^{-1} and k_s of 1.19 g/l. The carotenoid content related to the residence time appeared to assume a typical form of saturation kinetics. The maximum carotenoid content (X_m) for the mutant was estimated to be 1.04 μg/mg dry cell weight, and the Lee constant (k_m), which was tentatively defined in this work, was found to be 3.0 h.

Keywords: Carotenoids, residence time, maximum specific growth rate constant, Monod constant, Lee constant, *Xanthophyllomyces dendrorhous*

Xanthophyllomyces dendrorhous (formerly known as *Phaffia rhodozyma*) is known as a carotenoid-producing red yeast that synthesizes astaxanthin as its main carotenoid (85% of the total carotenoid content) [2, 10, 21, 27]. Astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione), which can also be produced by green alga *Haematococcus pluvialis* [9, 25], is used as a red pigmentation source in aquaculture, primarily in salmon and trout [17], and has important applications in the nutraceutical and food industries because of its possible role in human health and nutrition [7, 17].

However, since the current cost of astaxanthin is US$ 2,500/kg [18], this restricts its extensive use in foods and as a feed additive. Thus, various attempts have been made to reduce its production costs, including the use of less expensive carbon sources [8, 11, 22], optimizing the process conditions [12–14], and strain development and genetic modification of the astaxanthin-biosynthetic pathway in *X. dendrorhous* [2, 4–6, 16, 27].

In a previous work by the present authors, a β-ionone-resistant mutant, *X. dendrorhous* SKKU 0107, was isolated from the wild-type red yeast *X. dendrorhous* KCTC 7704 [23]. The growth characteristics, fermentation kinetics with various carbon sources, and the effect of pH on the growth and carotenogenesis of *X. dendrorhous* SKKU 0107 were all studied, and the mutant found to yield up to a 2.3-fold higher carotenoid content compared with the wild-type strain. Therefore, the present study investigated the batch and continuous culture kinetics of carotenoid production by *X. dendrorhous* SKKU 0107 to identify the relationship between the degree of the physical maturation process and the carotenoid content in the yeast cells. In addition, two important kinetic parameters in the Monod equation [24] were estimated using steady-state data from continuous culture experiments. Although various reports exist on the strain development of *X. dendrorhous* and its optimized culture conditions, little information is available on how the degree of physical maturation of the yeast cells affects the carotenoid content in the cells. Therefore, this would appear to be the first report on the kinetic constants for growth and carotenogenesis in *X. dendrorhous*.

Strain *X. dendrorhous* SKKU 0107 was used throughout the course of this study and maintained through being

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transferred to fresh YM (1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone) agar plates at 4°C and subcultured biweekly. Single yeast colonies were taken from a stock culture plate and transferred to a sterilized Erlenmeyer flask containing 50 ml of the YM medium. After 3 days of shaking at 20°C, the yeast was transferred to a culture vessel, containing 950 ml of a fermentation medium in a 1-l fermentor (Kobiotech, Korea). Unless otherwise specified, the fermentation medium consisted of 1% glucose, 0.1% yeast extract (Difco), 0.5% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.01% $CaCl_2 \cdot 2H_2O$. In addition, 0.05 ml of Neorin-400 per liter of the medium was used as an antifoam agent. The temperature was controlled at 22°C and the pH was maintained at 4.5. The aeration and agitation were 0.5 vvm and 300 rpm, respectively. The dissolved oxygen tension (DOT) was monitored using a dissolved oxygen probe (Mettler-Toledo GmbH, Germany) and the DOT level was maintained above 20% during the course of the fermentation.

For the glucose-limited continuous culture kinetic studies, only the glucose concentration in the fermentation medium was decreased from 1% to 0.5%. To allow steady-state conditions to be reached in all the experiments, at least 5–6 working volumes of the fermentation medium were replaced in the fermentor. The cell growth was measured in terms of the absorbance at 600 nm. The dry cell weights (DCWs) were determined for 10-ml samples that were washed with 10 ml of NaCl (0.85% solution) and once with distilled water, and then dried at 105°C for 20 h. The estimation of glucose was performed using the dinitrosalicylic acid method [20]. The extraction and quantitative analyses of the carotenoids from *X. dendrorhous* SKKU 0107 were carried out as described by Sedmak *et al.* [25] with minor modifications: One ml of the culture broth was used, and the cells washed twice with water. After decanting the water, the cells were then treated with 2 ml of dimethyl sulfoxide (DMSO) at 55°C for 5 min. Next, 0.1 ml of 0.01 M sodium phosphate (pH 7.0) was added to a tube containing the DMSO-treated cells, and then the mixture was vortexed for 30 sec. Thereafter, 2 ml of ethylacetate: hexane 50:50% (v/v) was added, and the tube was vortexed again for 30 sec, centrifuged at 10,000 ×*g* for 15 min, and the pigment-containing upper phase was recovered. The total carotenoids were estimated at 480 nm using a spectrophotometer (Hewlett Packard 8453, Germany), with authentic astaxanthin (Sigma) as the standard [3, 25].

Batch Culture Kinetics of *X. dendrorhous* SKKU 0107

The characteristics of the β-ionone-resistant *X. dendrorhous* SKKU 0107, which was isolated from 0.1 mM of β-ionone by using NTG mutagenesis, are described in a previous paper by the present authors [23]. The strain remained resistant with up to 0.15 mM of β-ionone, although the parent strain KCTC 7704 was strongly inhibited at

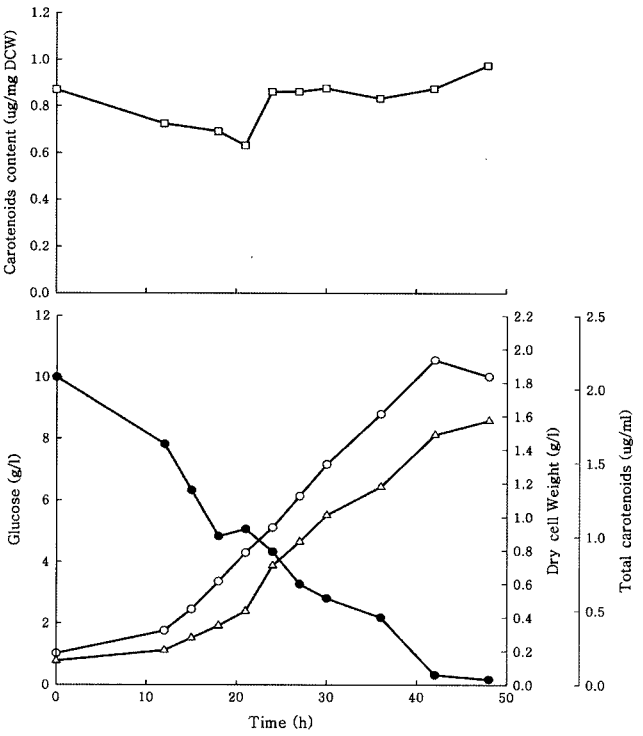

Fig. 1. Batch culture kinetics for *X. dendrorhous* SKKU 0107 on 10 g/l glucose medium at 22°C and pH 4.5.
Symbols: glucose (●), dry cell weight (○), total carotenoids (△), and carotenoid content (□).

0.025 mM. Fig. 1 shows the batch culture kinetics of *X. dendrorhous* SKKU 0107 on a 10 g/l glucose medium at 22°C and pH 4.5. The glucose concentrations declined slowly with the fermentation time, and the fermentation was complete after 48 h. At this time, the final cell (biomass) concentration was 1.9 g/l, and the cell yield was 0.2 g/g glucose consumed. As seen from Fig. 1, the concentration of carotenoids did not coincide with the increase in biomass. Similar results, known as mixed-growth-associated product formation, have also been reported previously [1, 10, 13, 19]. Yeasts divide by budding on their own growth, and it is not difficult to distinguish between the mother and daughter cells. Thus, a growing population of yeast cells has a changing cell-age distribution. In *H. pluvialis*, astaxanthin biosynthesis has been associated with a remarkable change from vegetative to cyst cells and a maturation (immature to mature cyst cells) process involved in the accumulation of astaxanthin in the alga [25]. Similarly, the content of intracellular carotenoids in *X. dendrorhous* depended on the degree of physical maturation toward adulthood, which would help explain the mixed-growth-associated product formation described above.

The carotenoid content in *X. dendrorhous* during the course of the batch fermentation is illustrated in Fig. 1. The content at the beginning of the fermentation was the same as that in the inoculum, and the current study used an

Table 1. Growth and carotenoid production by *X. dendrorhous* SKKU 0107 on 10 g/l glucose medium after 4 days at different temperatures.

Kinetic parameter	Temperature (°C)			
	20	22	24	26
Dry cell weight (mg/ml)	2.1	1.9	1.5	-
Cell yield (mg/mg glucose)	0.21	0.20	0.17	-
Carotenoid content (μg/mg dry cell weight)	0.69	0.87	0.82	-
Total carotenoids (μg/ml)	1.45	1.68	1.23	-

inoculum of 3-day-cultured yeast cells with a carotenoid content of 0.87 μg/mg dry cell weight. During the early period of fermentation (10–20 h), relatively younger yeast cells were dominant in the culture. Therefore, the carotenoid content was found to decrease to some extent until 20 h of fermentation, beyond which the content increased owing to the steady increase in adult yeast cells. Thus, it would seem that the yeast cells required about 20–30 h for their physical maturation.

Effect of Temperature on Growth and Carotenoid Production

Table 1 summarizes the effect of temperature on the fermentation kinetic parameters. As the fermentation temperature increased from 20 to 26°C, the final biomass (dry cell weight) tended to decrease. Unlike other reported results [10, 19], no appreciable growth occurred at 26°C. When compared with the kinetic parameters at 22°C, a lower carotenoid content was evident at 20°C and a reduced biomass at 24°C. The overall optimum temperature for growth and fungal carotenogenesis was found to be 22°C, which agrees with the results from other studies obtained by other authors [10, 13, 19]. Therefore, a temperature of 22°C was selected for the remaining experiments with *X. dendrorhous* SKKU 0107.

Continuous Culture with *X. dendrorhous* SKKU 0107

Unfortunately, little work has been done on the continuous culture kinetics of *X. dendrorhous* [19, 26]. Thus, to estimate the maximum specific growth rate constant (μ_m) and Monod constant (k_s), continuous-culture experiments were conducted with *X. dendrorhous* SKKU 0107 using a 5 g/l glucose medium at 22°C and pH 4.5. At low dilution rates, the glucose was fully metabolized, as shown by the steady-state data in Fig. 2. However, increasing the dilution rate resulted in an increased remaining glucose concentration and decreased biomass concentration. At steady state, the Monod equation [24] becomes

$$1/D=(k_s/\mu_m)\cdot 1/S+1/\mu_m \quad (1)$$

where S is the steady-state glucose concentration (g/l) and D is the dilution rate (h^{-1}). A plot of 1/D against 1/S should give a straight line with a slope of k_s/μ_m and an intercept on the ordinate at $1/\mu_m$. From Fig. 3, the values of μ_m and k_s were estimated to be 0.15 h^{-1} and 1.19 g/l, respectively. The μ_m value was found to be comparable to those

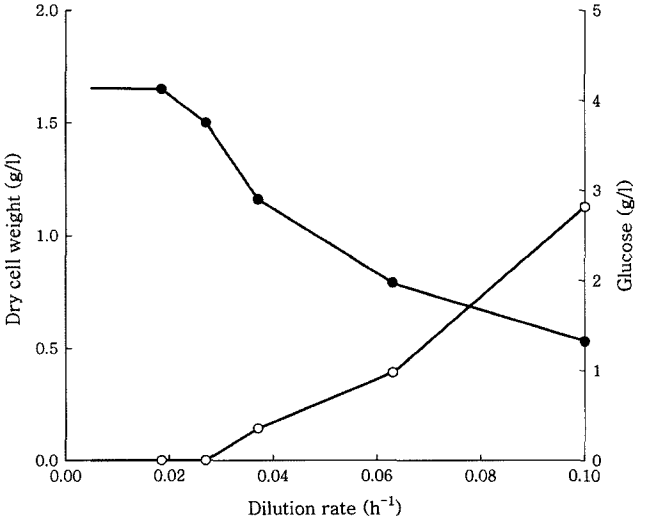

Fig. 2. Continuous culture with *X. dendrorhous* SKKU 0107 using 5 g/l glucose medium at 22°C and pH 4.5.
Symbols: glucose (●) and dry cell weight (○).

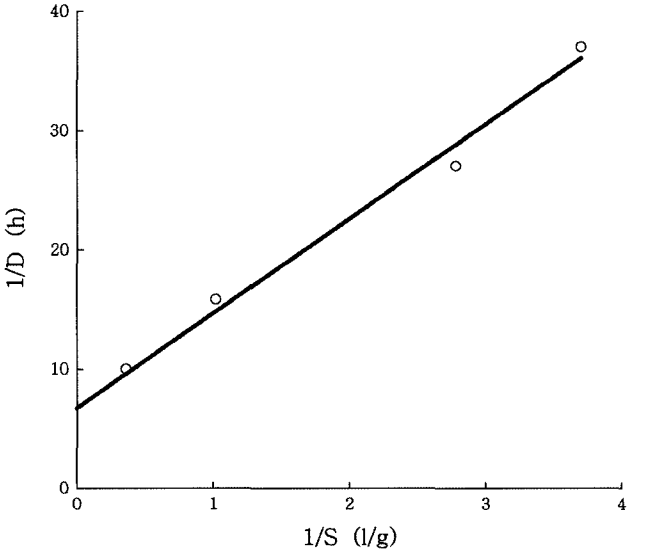

Fig. 3. Plot for reciprocal of dilution rate (1/D) versus reciprocal of remaining glucose concentration (1/S).

reported previously [10, 19]. As no k_s value is available for *X. dendrorhous* in existing literature, it was not possible to compare the present k_s value with others. Therefore, this would appear to be the first reported value for *X. dendrorhous*. However, the typical values of k_s for the growth of organisms are normally quite small. For example, a k_s value of 0.025 g/l was previously reported for the yeast *Saccharomyces cerevisiae* [24]. Thus, the relatively high value of k_s for *X. dendrorhous* SKKU 0107 requires some explanation. One possible reason is that *X. dendrorhous* cells are more rigid than *S. cerevisiae* cells. Unlike *S. cerevisiae*, it was much more difficult to take out the red yeast colonies from a stock culture plate because of the relative hardness of the *X. dendrorhous* cells. This may provide a significantly increased diffusional resistance as regards the uptake of glucose by *X. dendrorhous*.

Effect of Residence Time on Carotenoid Content of *X. dendrorhous*

As shown in Fig. 1, it appeared that the *X. dendrorhous* cells required about 20–30 h for their physical maturation process. Accordingly, the degree of carotenogenesis in *X. dendrorhous* was investigated in terms of the residence time, which is the reciprocal of the dilution rate. Fig. 4 shows the effect of the residence time (*i.e.*, aging time) on the carotenoid content in the *X. dendrorhous* cells. As seen from Fig. 4, the carotenoid content (X) relative to the residence time (T) appeared to follow a typical form of saturation kinetics:

$$X=X_m \cdot T/(k_m+T) \quad (2)$$

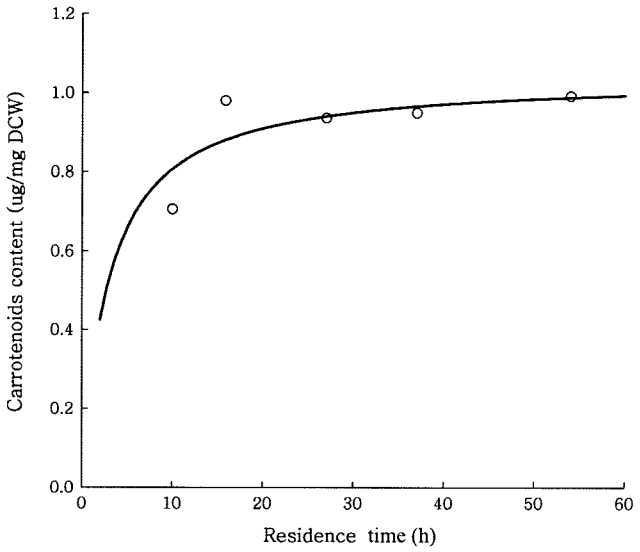

Fig. 4. Effect of residence time on the carotenoid content in *X. dendrorhous* SKKU 0107 cells.
Symbols: experimental data (○) and "best fit" curve obtained with a computer program (-).

where X_m is the maximum carotenoid content when $T \gg k_m$, and k_m is tentatively defined as a Lee constant in this work and equal to the residence time when the content is equal to one-half of the maximum value. These two values may be strain-dependent. Therefore, based on the results shown in Fig. 4, the maximum carotenoid content (X_m) and Lee constant (k_m) value for *X. dendrorhous* SKKU 0107 were graphically determined as 1.04 μg/mg dry cell weight and 3.0 h, respectively.

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